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(54) Title: HUMANISED ANTIBODIES AND USES THEREOF

(57) Abstract: A humanised antibody capable of binding to the MUC1 mucin antigen comprises a light chain and a heavy chain. The variable region of the light chain (V_L) comprising an amino acid sequence which is substantially homologous with the sequence of Fig.1A and the variable region of the heavy chain (V_H) comprising an amino acid sequence which is substantially homologous with the sequence of Fig. 1B. The amino acid residue at position 46 on V_L is backmutated to arginine, and the amino acid residue at position 47 on V_H is backmutated to leucine. The humanised antibody has use in the diagnosis and/or treatment of cancer.

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HUMANISED ANTIBODIES AND USES THEREOF

INTRODUCTION

The invention relates to a humanised version of the murine C595 antibody, and to uses of the humanised antibody in the diagnosis, staging and treatment of cancers.

The MUC1 mucin is expressed by secretory epithelia. Its abberant glycosylation in tumours allows it to be exploited as a marker for antibody targeted diagnosis and therapy. The C595 murine monoclonal antibody targets the epitope Arg-Pro-Ala-Pro on the MUC1 protein core. It has been used both *in-vitro* and *in-vivo* in the diagnosis of breast and bladder cancer. A phase 1 clinical trial of the antibody as a radioimmunotherapeutic agent in bladder cancer by intravesical administration has recently been initiated. Its potential use as an intravenous diagnostic has been limited by its murine origin.

It is an object of the invention to overcome this problem.

STATEMENTS OF INVENTION

Accordingly, the invention provides a humanised antibody capable of binding to the MUC1 mucin antigen comprising a light chain and a heavy chain, the variable region of the light chain (V_L) comprising an amino acid sequence which is substantially homologous with the sequence of Fig.1A, the variable region of the heavy chain (V_H) comprising an amino acid sequence which is substantially homologous with the sequence of Fig.1B wherein the amino acid residue at position 46 on V_L is backmutated to arginine, and wherein the amino acid residue at position 47 on V_H is backmutated to leucine. The V_L domain is joined to the human immunoglobulin Kappa constant domain to form the complete light chain. Similarly, the V_H domain is joined to the human immunoglobulin gamma-1 constant domains to form the complete heavy chain.

In this specification the term "substantially homologous" should be understood as meaning that the degree of homology is sufficient to allow binding to the MUC1 mucin antigen when any of the various backmutation combinations of the invention are included. Thus, stated another way, the antibodies according to the invention comprise a light chain and a heavy chain, the V_L domain of the light chain comprising a framework region (FR) derived from the Bence Jones protein REI and complementarity-determining regions (CDR) derived from the murine C595 antibody, the FR including at least one backmutation at position 46 to arginine, the V_H domain of the heavy chain comprising a FR derived from myeloma protein HIL and CDR derived from murine C595 antibody, the FR including at least one backmutation at position 47 to leucine.

Typically, the V_L domain will have at least a 60%, preferably at least 70%, more preferably at least 80%, more preferably at least 90%, and most preferably at least 95% homology with the amino acid sequence of Fig.1A

Similarly, the V_H domain will typically have at least a 60%, preferably at least 70%, more preferably at least 80%, more preferably at least 90%, and most preferably at least 95% homology with the amino acid sequence of Fig.1B.

Preferably, the V_L domain will include further backmutations to improve binding affinity. In one embodiment of the invention the amino acid residue at position 4 of the V_L domain is backmutated to leucine.

Preferably, the amino acid residues at positions 4 and 1 of the V_L domain are backmutated to leucine and glutamine respectively. Ideally, the amino acid residues at positions 4, 1 and 47 on the V_L domain are backmutated to leucine, glutamine and tryptophan respectively. The combination of these three backmutations with the backmutation on residue 46 of the V_L domain has the effect of increasing the affinity of the humanised antibody for the antigen seven-fold. Suitably, the amino acid residues at positions 4, 1, 47 and 3 on the V_L domain are backmutated to leucine, glutamine, tryptophan and valine respectively. Typically, the amino acid residues at positions 4, 1, 47, 3,

40 and 70 on the V_L domain may be backmutated to leucine, glutamine, tryptophan, valine, serine and serine respectively.

In another embodiment of the invention, the amino acid residues at positions 4 and 47 on the V_L domain are backmutated to leucine and tryptophan. In a further embodiment of the invention the amino acid residue at position 47 on the V_L domain is backmutated to tryptophan. In a still further embodiment of the invention, the amino acid residues at positions 1, 3 and 4 on the V_L domain are backmutated to glutamine, valine and leucine.

The possible permutations for back mutations to the V_L domain according to the invention is summarised in Table 2A.

Preferably, the V_H domain will include further backmutations. Thus, for example, the backmutation of the amino acid residue at position 42 on the V_H domain to aspartic acid has been found to increase the binding affinity of the antibody two-fold. Furthermore, the backmutation of the amino acid residue at position 16 on the V_H domain to glycine has been demonstrated to reduce the non-specific binding of the antibody to other unrelated antigens. The possible backmutation permutations of the V_H domain according to the invention are summarised in Table 2B.

Most preferably, the humanised antibody comprises the backmutation indicated as BMLr in Table 2A and the backmutation indicated as BMHq in Table 2B.

The V_L domain according to the invention typically comprises a framework region (FR) and complementarity determining regions (CDR), wherein the FR region is derived from the Bence Jones protein REI, and wherein the CDR is obtained from the C595 antibody.

The V_H domain according to the invention typically comprises a framework region (FR) and complementarity determining regions (CDR), wherein the FR region is derived from the myeloma protein HIL, and wherein the CDR is obtained from the C595 antibody.

In a preferred embodiment of the invention, the humanised antibody according to the invention is conjugated to a radioactive isotope. Ideally, the

radioactive isotope is selected from the group of Technetium-99m, Rhenium-188, Copper-67 and Indium-111.

The invention also relates to the use of a humanised antibody according to the invention in the diagnosis and/or treatment of cancer, in the intravesical diagnosis and/or therapy of bladder tumour and/or bladder cancer, in the intravenous diagnosis, staging and/or therapy of metastatic bladder cancer, and in the intravenous diagnosis and/or therapy of localised and/or metastatic cancers expressing the MUC1 mucin antigen, especially bladder, breast and ovarian cancers.

The invention also relates to a variable light chain domain (V_L) for a humanised antibody according to the invention comprising an amino acid sequence which has a sufficient degree of homology with the sequence of Fig.1A to allow binding to the MUC1 mucin antigen when one of the backmutation combinations given in Table 2A is included.

The invention also relates to a variable heavy chain domain (V_H) for a humanised antibody according to the invention and comprising an amino acid sequence which has a sufficient degree of homology with the sequence of Fig.1B to allow binding to the MUC1 mucin antigen one of the backmutation combinations given in Table 2B is included.

The invention also relates to the use of the V_L domain and/or the V_H domain of the invention in the formation of a humanised antibody and/or an antibody binding fragment (e.g. single chain FV antibody, diabody, and other multivalent derivatives) which is capable of binding to the MUC1 mucin antigen.

The invention also seeks to provide a method for the treatment or diagnosis of cancer, comprising administering an effective amount of a humanised antibody according to the invention to a patient.

The invention also provides a humanised antibody according to the invention for use in the manufacture of a medicament for the treatment or diagnosis of cancer.

DETAILED DESCRIPTION OF THE INVENTION

Preparation of human framework regions for CDR grafting:

The framework regions (FRs) from the Bence-Jones protein REI [V_L, Protein databank [PDB] access code: 1REI, Kabat subgroup (Kabat *et al.*, 1991): human kappa I] and the myeloma protein HIL (V_H, PDB access code: 8FAB, Kabat subgroup: human heavy III) were used as acceptor FRs for the CDRs from C595 in CDR grafting. A number of amino acid residues in these FRs were substituted by the consensus residue at those positions within the corresponding subgroup because of their relatively low occurrence in the subgroups and are therefore likely to have arisen from idiosyncratic mutations (table 1). These substitutions ensure that the human FRs represents human immunoglobulin sequences as a whole, rather than an individual sequence containing unnecessary mutations (which may only be useful for that particular antibody). All substituted residues are already present in the original murine C595 sequence and therefore such substitutions should not be detrimental to antigen binding. Tyr-71(V_L) was not substituted because it is positioned in the Vernier zone (Foote and Winter, 1992) of C595 V_L and may have important interactions with the CDRs.

Table 1. Residues in the FRs of (a) 1rei and (b) 8fab which deviate from the consensus sequence within their Kabat subgroups.

(A) 1rei (V_L) – human subgroup kappa I

Residue	Occurrence in Kabat subgroup (%)	Substitution by consensus (first letter = original residue number = Kabat residue number last letter = consensus substitution)
Thr-39	3	T39K
Tyr-71	3	No – Vernier zone residue
Phe-73	26	-
Ile-83	21	-
Leu-104	24	-
Thr-107	5	T107K

(B) 8fab (V_H) – human subgroup heavy III

Residue	Occurrence in Kabat subgroup (%)	Substitution by consensus (first letter = original residue number = Kabat residue number last letter = consensus substitution)
PCA*-1	12	PCA1E*
Lys-3	2	K3Q
Gln-6	6	Q6E
Ala-7	2	A7S
Val-11	25	-
Arg-16	28	-
Ile-23	2	I23A
Ala-49	30	-
Arg-76	2	R76N
Met-80	3	M80L
Thr-84	10	-
Val-107	2	V107T

* PCA = pyrollidone carboxylic acid

CDR grafting:

The finalised FRs were joined to CDRs from C595 to form the sequence BLC595a. The complete amino acid sequence of the BLC595a variable region is shown in figure 1. The DNA sequence for BLC595a was then deduced according to common codon usage for immunoglobulins (Kabat *et al*, 1991). To this DNA sequence, a cassette containing the recognition sequence for the restriction enzyme HindIII [(AAG:CTT) (other suitable restriction enzyme recognition sequences may also be used for subcloning into expression vectors)], the Kozak initiation sequence (Kozak, 1987) and an immunoglobulin signal peptide sequence from the antibody sharing the highest sequence homology with the corresponding humanised V_L and V_H domains (i.e. BLC595 V_L and V_H) published in the Kabat database (Kabat *et al.*, 1991) were added upstream. Also, a splice donor site (Bendig and Jones, 1996; optional

depending on the expression vectors used) and the recognition sequence for the restriction enzyme BamHI [(GGA:CTT), or other appropriate restriction enzyme recognition sequence] were added downstream to this sequence. This whole sequence (i.e. HindIII-Kozak-signal-BLC595 V_L/V_H-splice donor-BamHI; to be referred to as "the encoding sequence") for each of V_L and V_H was then analysed for the presence of internal splice donor and restriction sites (e.g. BamHI/HindIII) with the Genetics Computer Group (GCG) Wisconsin Package v.9.0. The complete DNA encoding sequences for BLC595a V_L and V_H are shown in figure 2.

The encoding sequences were synthesised *de novo* by the polymerase chain reaction (PCR). Eight overlapping oligonucleotide primers (each of around 80-nucleotide in length; figure 2) were synthesised to cover each of the V_L and V_H encoding sequences for BLC595a in a series of PCRs (Bendig and Jones, 1997; figure 3). The PCR products representing full length V_L and V_H were cloned and their sequences confirmed to yield the CDR-grafted sequence BLC595a.

PCR for BLC595a construction (Referring to Fig.3)

1) Reactions 1 and 2:

5μL	Geneamp 10x PCR buffer with 15mM $MgCl_2$ (Perkin-Elmer)
1μL	10mM dNTP Mix (Sigma)
12.5pmol	each of PL/H1, 2, 3, 4 (reaction 1 – V_L/V_H) or PL/H5, 6, 7, 8 (reaction 2 V_L/V_H)
2.5units	AmpliTaq DNA polymerase (Perkin Elmer) + sufficient sterilised, deionised water to 50μL

Conditions:
1) 94°C – 5 minutes (hot start)
2) 94°C – 2 minutes) x 8 cycles
72°C – 5 minutes)
3) 72°C – 10 minutes

2) Reactions 3, 4 and 6

5μL	Geneamp 10x PCR buffer with 15mM $MgCl_2$ (Perkin-Elmer)
1μL	10mM dNTP Mix (Sigma)
5μL	PCR product from reaction 1 (reaction 3, V_L/V_H), reaction 2 (reaction 4, V_L/V_H) or reaction 5 (reaction 6 - V_L/V_H)
40pmol each	PNLHA and PNLB2 (reaction 3, V_L) PNLHA and PNHB2 (reaction 3, V_H) PNLC2 and PNLD (reaction 4, V_L) PNHC2 and PNHD (reaction 4, V_H) PNLHE and PNLF (reaction 6, V_L) PNLHE and PNHF (reaction 6, V_H)
2.5units	AmpliTaq DNA polymerase (Perkin Elmer) + sufficient sterilised, deionised water to 50μL

Conditions:
1) 94°C – 5 minutes (hot start)
2) 94°C – 1.5 minutes)
64°C – 1.5 minutes) x 20 cycles
72°C – 2.5 minutes)
3) 72°C – 10 minutes

3) Reaction 5:

5μL	Geneamp 10x PCR buffer with 15mM $MgCl_2$ (Perkin-Elmer)
1μL	10mM dNTP Mix (Sigma)
5μL each	PCR products from reactions 3 and 4 (V_L/V_H)
2.5units	AmpliTaq DNA polymerase (Perkin Elmer) + sufficient sterilised, deionised water to 50μL

Conditions: 1) 94°C – 5 minutes (hot start)
2) 94°C – 2 minutes) x 8 cycles
72°C – 5 minutes)
3) 72°C – 10 minutes

Introduction of backmutations:

Backmutations are defined as the substitution of the amino acid residue at a position in the chosen human framework with the residue at the same position in the mouse antibody C595. These were introduced in an attempt to optimise the antigen binding ability of BLC595 after CDR grafting. Mutations were introduced by the method of overlap extension PCR (Higuchi *et al.*, 1988). All mutants were cloned and sequenced prior to antibody expression. A number of backmutants of V_L and V_H were made that incorporated one or more such amino acid backmutations. The positions for backmutations were determined initially on the common framework positions known to affect CDR conformations [namely, the Vernier zone (Foote and Winter, 1992), V_L/V_H interface (Chothia *et al.*, 1985), V_L N-terminal residues (Padlan, 1994) and putative O- and N-glycosylation syites (Bendig and Jones, 1997)]. These were exhausted before other backmutations were explored. In the case of BLC595, it was mainly the other backmutations, which were not obvious from previous publications, that led to a high level of restoration to specific MUC1 binding. Mutations in all the backmutants (represented by BMLx for V_L mutants and BMHx for V_H mutants) are shown in table 2 below.

Table 2. Mutations incorporated into the human frameworks. The first letter of each backmutation indicates the original amino acid residue in the human framework. The number indicates the amino acid position (Kabat numbering system; Kabat et al, 1991). The last letter indicates the new amino acid residue after backmutation.

(A) BLC595 V_L backmutants

Backmutant	Backmutations						
	D1Q	Q3V	M4L	P40S	L46R	L47W	D70S
BMLb	•	•	•	•	•	•	•
BMLc			•		•	•	
BMLd					•		
BMLg	•	•	•		•	•	
BMLj			•		•		
BMLm					•	•	
BMLn	•	•	•		•		
BMLp	•		•		•		
BMLq		•	•		•		
BMLr	•		•		•	•	

(B) BLC595 V_H backmutants:

Back mutant	V11 L	R16G	R19K	A40T	G42D	G44R	W47L	S74A	N(82A)S	R83K	T84S	V89M	L108T	V109L
BMHb				•			•		•				•	
BMHc							•							
BMHe					•	•	•							
BMHf							•	•				•		
BMHg							•						•	•
BMHi							•			•	•			
BMHj							•							
BMHk	•	•	•				•							
BMHm					•		•							
BMHn						•	•							
BMHp	•				•		•							
BMHq		•			•		•							
BMHr			•		•		•							

Final BLC595 sequence and antibody expression.

The final BLC595 variable region consists of the backmutants BMLr and BMHq. The complete amino acid sequences are shown in figure 4. The encoding sequences for BMLr and BMHq were excised from the cloning vector by appropriate restriction digests and were subcloned into expression vectors containing the human constant regions kappa and gamma-1 respectively for whole IgG expression (for example, pKN10 – light chain; pG1D16/20 – heavy chain – from Medical Research Council Technology). These BLC595 expression vectors (for example, 10μg each of pKN10-BLC595 V_L and pG1D16/20 - BLC595 V_H) were then co-transfected into $7x10^6$ COS-7 cells by electroporation at 1900V, 25μF. Cells were then transferred to 8mLs of pre-warmed medium (Dulbecco modified eagle medium supplemented with 10% (v/v) ultra low IgG-foetal bovine serum, 580 μg/ml L-glutamine and 50 Units/ml penicillin / 50 μg/ml streptomycin). Antibodies were harvested in the medium 48-72 hours post transfection. Purified BLC595 was obtained by standard Sepharose-protein A affinity chromatography.

Methods for Radiolabelling of Antibodies

We envisage the use of ^{99m}Tc (or other gamma-emitting isotopes) as a diagnostic radionuclide and ^{188}Re (or other gamma- and beta-emitting isotopes) as a diagnostic/ therapeutic radionuclide for BLC595. Labelling of antibodies with these radioisotopes are available in the literature and references are given below:

1) Technetium-99m:

Pimm MV, Gribben SJ (1993) Radiolabelling antibodies for imaging and targeting. In: Tumour Immunobiology; A Practical Approach (Gallagher, Rees & Reynolds, eds) pp 209-223. Oxford University Press. (also for rhenium-188)

Mather SJ & Ellison D (1990) Reduction mediated technetium-99m labelling of monoclonal antibodies. *J. Nucl. Med* 31: 692-697.

2) Rhenium-188:

Griffiths GL, Goldenberg DM, Diril H & Hansen HJ (1994) Technetium-99m, Rhenium-186 and Rhenium-188 direct-labeled antibodies. *Cancer* 73: 761-768.

Potential Usage of BLC595-based Radiopharmaceuticals

Superficial Bladder Cancer: Intravesical Administration

The antibody can be utilised via the intravesical administration of BLC595 conjugated to radioactive isotopes to detect the presence of MUC1 mucin positive tumour cells within the confines of the bladder. Radionuclides include both ^{67}Cu and ^{99m}Tc for diagnostic purposes. Allied to the use of ^{99m}Tc is the isotope ^{186}Re, which has similar chemical characteristics to ^{99m}Tc but with a appropriate beta emission for cellular cytotoxicity and as such can be exploited in a therapeutic context. In a similar manner ^{67}Cu can be used in both a diagnostic and therapeutic scenario (it has both gamma and beta energy emission) although routine use of ^{67}Cu would be limited because it is not readily available widely.

Bladder Cancer: Invasive and Metastatic Disease

The same arguments apply for the use of BLC595 by systemic administration in the diagnosis and the treatment of metastatic bladder cancer. In human bladder cancer, we are not aware of the use of similar approaches using other radiolabelled anti-MUC1 mucin monoclonal antibodies. The humanised nature of BLC595 allow it to be administered repeatedly in multiple dosing regimens, whilst keeping the likelihood of human anti-mouse antibody (HAMA) response to a minimum. As a diagnostic and disease staging tool, preliminary data has shown that systemic use of the parent antibody C595 coupled to ^{111}In, ^{67}Cu, ^{99m}Tc and ^{186}Re would have the potential to be as useful as, if not better than, magnetic resonance imaging in instances where metastatic disease expresses MUC1. In the same way we would see therapeutic doses of radiolabelled antibody being utilised to treat patients of their disease.

Ovarian Cancer

Pre-clinical and clinical evaluation of the use of BLC595-based radioimmunoconjugates in the bladder cancer model should lead to their application in other diseases where MUC1 tumour expression is well characterised. This includes breast and ovarian carcinomas. In an ovarian study, we would use our reagents in diagnosis by their administration into the peritoneum. Because of the involvement of the hosts immune system in this cavity, the humanised antibody conjugate would offer the greatest chance of evading the HAMA response. Multiple administration for potential therapeutic effect could therefore be envisaged. Metastatic ovarian cancers may also be detected and treated in the same manner as metastatic bladder cancer using BLC595 conjugated to the aforesaid radionuclides.

Metastatic Breast Cancer

We could also see BLC595 finding a suitable role in the diagnosis and possible management of breast cancer. This again would involve systemic administration of the radioimmunoconjugate.

Current Phase I/II Trials

Our use of ^{67}Cu labelled C595 in a diagnostic context has been published. We now have approval from the Cancer Research Campaign (CRC) to begin a Phase I clinical trial in human bladder cancer using ^{67}Cu -labelled C595 administered intravesically. Phase II trails using similar protocols should commence upon the completion of this study. This should ascertain the clinical utility of our radioimmunoconjugate (proof of principle) and should lead to similar trials being set up using ^{188}Re labelled C595, a more widely available radionuclide and therefore more commercially viable. Similar studies with radiolabelled BLC595 would follow after appropriate preclinical evaluation. The way forward into the systemic usage of this antibody would then be forged, so that experimentation on disseminated disease can progress. The use of appropriate higher does of this radioimmunoconjugate would see the use of this reagent in a potential therapeutic context.

The invention is not limited to the embodiments hereinbefore described which may be varied in construction and detail without departing from the spirit of the invention.

References

Bendig MM, Jones ST (1996) Rodent to human antibodies by CDR grafting. IN: McCafferty J, Hoogenboom, HR, Chiswell DJ (eds) *Antibody engineering – a practical approach*. NEW YORK: Oxford University Press.

Chothia C, Novotny J, Bruccoleri R, Karplus M (1985) Domain association in immunoglobulin molecules – the packing of variable domains. *J Mol Biol* **186**:651-663.

Foote J, Winter G (1992) Antibody framework residues affecting the conformation of the hypervariable loops. *J Mol Biol* **224**:487-499

Higuchi R, Krummel B, Saiki RK (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. *Nucleic Acids Res* **16**:7351-7367

Kabat EA, Wut TT, Perry HM, Gottesman KS, Foeller C (1991) Sequences of proteins of immunological interest. 5th edition. BETHESDA: US Department of Health and Human Services.

Kozak M (1987) At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J Mol Biol* **196**:947-950

Padlan EA (1994) Anatomy of the antibody molecule. *Mol Immunol* **31**(3):169-217.

CLAIMS

1. A humanised antibody capable of binding to a MUC1 mucin antigen comprising a light chain and a heavy chain, the variable region of the light chain (V_L) comprising an amino acid sequence which is substantially homologous with the sequence of Fig.1A, the variable region of the heavy chain (V_H) comprising an amino acid sequence which is substantially homologous with the sequence of Fig.1B, wherein the amino acid residue at position 46 on V_L is backmutated to arginine, and wherein the amino acid residue at position 47 on V_H is backmutated to leucine.

2. A humanised antibody as claimed in claim 1 in which the amino acid residue at position 4 of V_L is backmutated to leucine.

3. A humanised antibody as claimed in claim 2 in which the amino acid residue at position 1 of V_L is backmutated to glutamine.

4. A humanised antibody as claimed in claim 3 in which the amino acid residue at position 47 on V_L is backmutated to tryptophan.

5. A humanised antibody as claimed in claim 4 in which the amino acid residue at position 3 on V_L is backmutated to valine.

6. A humanised antibody as claimed in claim 5 in which the amino acid residues at positions 40 and 70 on V_L are backmutated to serine.

7. A humanised antibody as claimed in claim 1 in which the amino acid residue at position 47 on V_L is backmutated to tryptoptian.

8. A humanised antibody as claimed in claim 2 in which the amino acid residue at position 3 on V_L is backmutated to valine.

9. A humanised antibody as claimed in claim 3 in which the amino acid residue at position 47 on V_L is backmutated to tryptophan.

10. A humanised antibody as claimed in any preceding claim in which the amino acid residue at position 42 on V_H is backmutated to aspartic acid.

11. A humanised antibody as claimed in claim 10 in which the amino acid residue at position 16 on V_H is backmutated to glycine.

12. A humanised antibody as claimed in claim 10 in which the amino acid residue at position 44 on V_H is backmutated to arginine.

13. A humanised antibody as claimed in claim 10 in which the amino acid residue at position 11 on V_H is backmutated to leucine.

14. A humanised antibody as claimed in claim 10 in which the amino acid residue at position 19 on V_H is backmutated to lysine.

15. A humanised antibody as claimed in any of claims 1 to 9 in which the amino acid residues at positions 11, 16 and 19 on V_H are backmutated to leucine, glycine and lysine respectively.

16. A humanised antibody as claimed in any of claims 1 to 9 in which the amino acid residues at positions 40, 82a and 108 on V_H are backmutated to threonine, serine and threonine respectively.

17. A humanised antibody as claimed in any of claims 1 to 9 in which the amino acid residue at position 74 on V_H is backmutated to alanine.

18. A humanised antibody as claimed in any of claims 1 to 9 in which the amino acid residue at position 89 on V_H is backmutated to methionine.

19. A humanised antibody as claimed in any of claims 1 to 9 in which the amino acid residues at positions 108 and 109 on V_H are backmutated to threonine and leucine respectively.

20. A humanised antibody as claimed in any of claims 1 to 9 in which the amino acid residue at positions 83 and 84 on V_H are backmutated to lysine and serine respectively.

21. A humanised antibody as claimed in any preceding claim in which the V_L domain comprises a framework region (FR) and complementarity determining regions (CDR), wherein the FR region comprises the Bence Jones protein REI, and wherein the CDR are obtained from C595 antibody.

22. A humanised antibody as claimed in any preceding claim in which the V_H domain comprises a framework region (FR) and complementarity determining regions (CDR), wherein the FR region comprises the myeloma protein HIL, and wherein the CDR are obtained from C595 antibody.

23. A humanised antibody as claimed in any preceding claim conjugated to a radioactive isotope.

24. A humanised antibody as claimed in claim 23 in which the radioactive isotope is selected from the group of Technetium-99m, Rhenium-188, Copper-67 and Indium-111.

25. Use of a humanised antibody as claimed in any preceding claim in the diagnosis and/or treatment of cancer.

26. Use of a humanised antibody as claimed in any preceding claim in the intravesical diagnosis and/or therapy of bladder tumour and/or bladder cancer.

27. Use of a humanised antibody as claimed in any preceding claim in the intravenous diagnosis, staging and/or therapy of metastatic bladder cancer.

28. Use of a humanised antibody as claimed in any preceding claim in the intravenous diagnosis and/or therapy of localised and/or metastatic cancers expressing the MUC1 mucin antigen, especially bladder, breast and ovarian cancers.

29. A variable light chain domain (V_L) for a humanised antibody according to any of claim 1 to 22 comprising an amino acid sequence which has a sufficient degree of homology with the sequence of Fig.1A to allow binding to the MUC1 mucin antigen when one of the backmutation combinations given in Table 2A is included.

30. A variable heavy chain domain (V_H) for a humanised antibody according to any of claims 1 to 22 and comprising an amino acid sequence which has a sufficient degree of homology with the sequence of Fig.1B to allow binding to the MUC1 mucin antigen when one of the backmutation combinations given in Table 2B is included.

31. Use of the V_L domain of claim 29 and/or the V_H domain of claim 30 in the formation of a humanised antibody and/or an antibody binding fragment which is capable of binding to the MUC1 mucin antigen.

32. A method for the treatment or diagnosis of cancer, comprising administering an effective amount of a humanised antibody according to any of claims 1 to 24 to a patient.

33. A humanised antibody according to any of claims 1 to 24 for use in the manufacture of a medicament for the treatment or diagnosis of cancer.

34. A nucleic acid sequence which codes for any of the humanised antibodies of claims 1 to 22 or either of the V_L domain of claim 29 or V_H domain of claim 30.

FIG. 1A HUMANISED ANTIBODY BLC595a (No backmutations) V_L PRIMARY SEQUENCE INFORMATION

1	2	3	4	5	6	7	8	9	10	11	12
D	I	Q	M	T	Q	S	P	S	S	L	S
13	14	15	16	17	18	19	20	21	22	23	24
A	S	V	G	D	R	V	T	I	T	C	S
25	26	27	29	30	31	32	33	34	35	36	37
A	S	S	S	V	S	Y	M	H	W	Y	Q
38	39	40	41	42	43	44	45	46	47	48	49
Q	K	P	G	K	A	P	K	L	L	I	Y
50	51	52	53	54	55	56	57	58	59	60	61
D	T	S	K	L	A	S	G	V	P	S	R
62	63	64	65	66	67	68	69	70	71	72	73
F	S	G	S	G	S	G	T	D	Y	T	F
74	75	76	77	78	79	80	81	82	83	84	85
T	I	S	S	L	Q	P	E	D	I	A	T
86	87	88	89	90	91	92	93	94	95	96	97
Y	Y	C	Q	Q	W	S	S	N	P	P	T
98	99	100	101	102	103	104	105	106	107		
F	G	Q	G	T	K	L	Q	I	K		

Length of Sequence : 106 amino acids

Human Framework : 1REI (Bence Jones protein), Human kappa chain group I Plus changes from table 1A

Complementarity Determining Regions (rectangles) : **CDRL1**: L24-34 (10) **CDRL2**: L50-56 (7) **CDRL3**: L89-97 (9)

(CDR definitions and numbering scheme are according to: Kabat *et al.*, 1991)

FIG. 1B HUMANISED ANTIBODY BLC595a (No backmutations) V_H PRIMARY SEQUENCE INFORMATION

1	2	3	4	5	6	7	8	9	10	11	12
E	V	Q	L	V	E	S	G	G	G	V	V
13	14	15	16	17	18	19	20	21	22	23	24
Q	P	G	R	S	L	R	L	S	C	A	A
25	26	27	28	29	30	31	32	33	34	35	36
S	G	F	T	F	S	**S**	**Y**	**G**	**M**	**S**	W
37	38	39	40	41	42	43	44	45	46	47	48
V	R	Q	A	P	G	K	G	L	E	W	V
49	50	51	52	52A	53	54	55	56	57	58	59
A	**T**	**I**	**N**	**S**	**N**	**G**	**G**	**S**	**T**	**Y**	**Y**
60	61	62	63	64	65	66	67	68	69	70	71
P	**D**	**S**	**V**	**K**	**G**	R	F	T	I	S	R
72	73	74	75	76	77	78	79	80	81	82	82A
D	N	S	K	N	T	L	Y	L	Q	M	N
82B	82C	83	84	85	86	87	88	89	90	91	92
S	L	R	T	E	D	T	A	V	Y	Y	C
93	94	95	96	97	98	99	100	100A	100B	100C	101
A	R	**D**	**R**	**D**	**G**	**Y**	**D**	**E**	**G**	**F**	**D**
102	103	104	105	106	107	108	109	110	111	112	113
Y	W	G	Q	G	T	L	V	T	V	S	S

Length of Sequence : 120 amino acids

Human Framework : 8FAB (Myeloma protein HIL), Closest to human heavy chain group III
Plus changes from table 1B

Complementarity Determining Regions (rectangles) : **CDRH1**: H31-35 (5)
CDRH2: H50-65 (17)
CDRH3: H95-102(11)

FIG. 2A V_L ENCODING SEQUENCE FOR BLC595a (No backmutations) (429 bps)

5'-AA TCG ATA CGC TCC AAG CTT GCC GCC ACC ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT GTC CAC TCC
3'-TT AGC TAT GCG AGG TTC GAA CGG CGG TGG TAC CCT ACC TCG ACA TAG TAG GAG AAG AAC CAT CGT TGT CGA TGT CCA CAG GTG AGG

GAT ATT CAG ATG ACC CAG TCT CCA TCC TCC CTG TCT GCA TCT GTA GGA GAT AGA GTC ACC ATC ACC TGC AGT GCC AGT TCA
CTA TAA GTC TAC TGG GTC AGA GGT AGG AGG GAC AGA CGT AGA CAT CCT CTA TCT CAG TGG TAG TGG ACG TCA CGG TCA AGT

AGT GTA AGT TAT ATG CAC TGG TAC CAG CAG AAA CCA GGC AAA GCT CCT AAA CTC CTG ATC TAT GAC ACA TCC AAA CTG GCT TCT GGA
TCA CAT TCA ATA TAC GTG ACC ATG GTC GTC TTT GGT CCG TTT CGA GGA TTT GAG GAC TAG ATA CTG TGT AGG TTT GAC CGA AGA CCT

GTC CCA TCA AGG TTC AGT GGC AGT GGG TCT GGG ACA GAT TAC ACT TTC ACC ATC AGC AGC CTG CAG CCT GAA GAT ATT GCA ACT TAT
CAG GGT AGT TCC AAG TCA CCG TCA CCC AGA CCC TGT CTA ATG TGA AAG TGG TAG TCG TCG GAC GTC GGA CTT CTA TAA CGT TGA ATA

TAC TGC CAG CAG TGG AGT AGT AAC CCG CCC ACG TTC GGT CAA GGG ACC AAG TTG CAG ATC AAA CGT AAG TGG ATC CAA TTA GCC GAG
ATG ACG GTC GTC ACC TCA TCA TTG GGC GGG TGC AAG CCA GTT CCC TGG TTC AAC GTC TAG TTT GCA TTC ACC TAG GTT AAT CGG CTC

T-3'
A-5'

PCR Primers

PL1: AA TCG ATA CGC TCC AAG CTT GCC GCC ACC ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT GTC
PL2: TCC TAC AGA TGC AGA CAG GGA GGA TGG AGA CTG GGT CAT CTG AAT ATC GGA GTG GAC ACC TGT AGC TGT TGC TAC CAA
PL3: TCC TCC CTG TCT GCA TCT GTA GGA GAT AGA GTC ACC ATC ACC TGC AGT GCC AGT TCA AGT GTA AGT TAT ATG CAC TGG
PL4: TTT GGA TGT GTC ATA GAT CAG GAG TTT AGG AGC TTT GCC TGG TTT CTG CTG GTA CCA GTG CAT ATA ACT TAC ACT TGA
PL5: AAA GCT CCT AAA CTC CTG ATC TAT GAC ACA TCC AAA CTG GCT TCT GGA GTC CCA TCA AGG TTC AGT GGC AGT GGG TCT
PL6: AGT TGC AAT ATC TTC AGG CTG CAG GCT GCT GAT GGT GAA AGT GTA ATC TGT CCC AGA CCC ACT GCC ACT GAA CCT TGA
PL7: CTG CAG CCT GAA GAT ATT GCA ACT TAT TAC TGC CAG CAG TGG AGT AGT AAC CCG CCC ACG TTC GGT CAA GGG ACC AAG
PL8: A CTC GGC TAA TTG GAT CCA CTT ACG TTT GAT CTG CAA CTT GGT CCC TTG ACC GAA CGT GGG
PNLHA: TCG ATA CGC TCC AAG CTT GCC GCC
PNLB2: GT GTC ATA GAT CAG GAG TTT AGG A
PNLC2: CCT AAA CTC CTG ATC TAT GAC ACA
PNLD : CTG GGC TAA TTG GAT CCA CTT ACG
PNLHE: CGC TCC AAG CTT GCC GCC ACC ATG
PNLF : TAA TTG GAT CCA CTT ACG TTT GAT

(Note: Underlined residues represent artificial sequences added to allow more efficient restriction digest at the recognition sequences immediately adjacent to these positions. They will not be present in the encoding sequence after subcloning into the expression vector.)

FIG. 2B V_H ENCODING SEQUENCE FOR BLC595a (No backmutations) (471 bps)

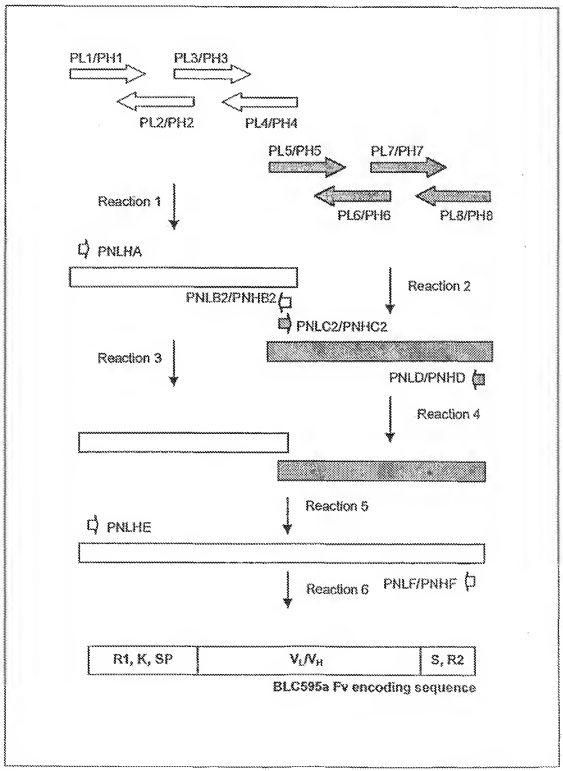
```
5'-AA TCG ACA CGC TCC AAG CTT GCC GCC ACC ATG GAG TTT GGG CTG AGC TGG CTT TTT CTT GTG GCT ATT TTA AAA GGT GTC CAG TGT
3'-TT AGC TAT GCG AGG TTC GAA CGG CGG TGG TAC CTC AAA CCC GAC TCG ACC GAA AAA GAA CAC CGA TAA AAT TTT CCA CAG GTC ACA
                ▼

GAG GTG CAG CTG GTG GAG TCT GGA GGA GGC GTG GTG CAG CCT GGG CGT TCA CTG AGA CTC TCC TGC GCA GCT TCT GGA TTC
CTC CAC GTC GAC CAC CTC AGA CCT CCT CCG CAC CAC GTC GGA CCC GCA AGT GAC TCT GAG AGG ACG CGT CGA AGA CCT AAG

ACC TTC AGT AGC TAC GGT ATG AGC TGG GTG CGC CAG GCT CCA GGA AAG GGC CTT GAG TGG GTC GCA ACC ATT AAT AGT AAT GGT GGT
TGG AAG TCA TCG ATG CCA TAC TCG ACC CAC GCG GTC CGA GGT CCT TTC CCG GAA CTC ACC CAG CGT TGG TAA TTA TCA TTA CCA CCA

AGC ACT TAC TAC CCA GAC TCT GTG AAG GGC CGA TTC ACA ATC TCC AGA GAC AAT TCC AAG AAC ACA CTG TAC CTG CAG ATG AAC AGC
TCG TGA ATG ATG GGT CTG AGA CAC TTC CCG GCT AAG TGT TAG AGG TCT CTG TTA AGG TTC TTG TGT GAC ATG GAC GTC TAC TTG TCG

CTG AGA ACT GAG GAC ACA GCC GTC TAT TAC TGT GCA AGA GAT AGG GAT GGT TAC GAT GAA GGT TTT GAC TAC TGG GGC CAA GGG ACC
GAC TCT TGA CTC CTG TGT CGG CAG ATA ATG ACA CGT TCT CTA TCC CTA CCA ATG CTA CTT CCA AAA CTG ATG ACC CCG GTT CCC TGG

CTG GTC ACC GTC TCC TCA GGT AAG TGG ATC CAA TTA GCC GAG T-3'
GAC CAG TGG CAG AGG AGT CCA TTC ACC TAG GTT AAT CGG CTC A-5'
```

PCR Primers

```
PH1: AA TCG ATA CGC TCC AAG CTT GCC GCC ACC ATG GAG TTT GGG CTG AGC TGG CTT TTT CTT GTG GCT ATT TTA AAA GGT GTC CAG
PH2: CAG TGA ACG CCC AGG CTG CAC CAC GCC TCC TCC AGA CTC CAC CAG CTG CAC CTC ACA CTG GAC ACC TTT TAA AAT AGC CAC
PH3: GTG GTG CAG CCT GGG CGT TCA CTG AGA CTC TCC TGC GCA GCT TCT GGA TTC ACC TTC AGT AGC TAC GGT ATG AGC TGG GTG
PH4: ACC ACC ATT ACT ATT AAT GGT TGC GAC CCA CTC AAG GCC CTT TCC TGG AGC CTG GCG CAC CCA GCT CAT ACC GTA GCT ACT
PH5: CTT GAG TGG GTC GCA ACC ATT AAT AGT AAT GGT GGT AGC ACT TAC TAC CCA GAC TCT GTG AAG GGC CGA TTC ACA ATC TCC
PH6: GTC CTC AGT TCT CAG GCT GTT CAT CTG CAG GTA CAG TGT GTT CTT GGA ATT GTC TCT GGA GAT TGT GAA TCG GCC CTT CAC
PH7: ATG AAC AGC CTG AGA ACT GAG GAC ACA GCC GTC TAT TAC TGT GCA AGA GAT AGG GAT GGT TAC GAT GAA GGT TTT GAC TAC
PH8: A GTC GGC TAA TTG GAT CCA CTT ACC TGA GGA GAC GGT GAC CAG GGT CCC TTG GCC CCA GTA GTC AAA ACC TTC ATC GTA ACC
PNLHA: TCG ATA CGC TCC AAG CTT GCC GCC
PNHE2: T ACT ATT AAT GGT TGC GAC CCA CT
PNHC2: GAG TGG GTC GCA ACC ATT AAT AGT
PNHD : CTC GGC TAA TTG GAT CCA CTT ACC
PNLHE: CGC TCC AAG CTT GCC GCC AGC AT
PNHF : TAA TTG GAT CCA CTT ACC TGA GGA
```

(Note: Underlined residues represent artificial sequences added to allow more efficient restriction digest at the recognition sequences immediately adjacent to these positions. They will not be present in the encoding sequence after subcloning into the expression vector.)

FIG. 3 *De novo* construction of BLC595a (No backmutations) by the Polymerase Chain Reaction



Key: Block arrows represent PCR primers (figure 2). R1=HindIII recognition sequence, K=Kozak initiation sequence, SP=immunoglobulin signal peptide sequence, V_L/V_H = BLC595a variable region sequences, S=splice donor sequence, R2=BamHI recognition sequence. (See text)

FIG. 4A **FINAL SEQUENCE (INCORPORATING BACKMUTATIONS) FOR HUMANISED ANTIBODY BLC595 V_L (BMLr)**

1	2	3	4	5	6	7	8	9	10	11	12
Q	I	Q	L	T	Q	S	P	S	S	L	S
13	14	15	16	17	18	19	20	21	22	23	24
A	S	V	G	D	R	V	T	I	T	C	S
25	26	27	29	30	31	32	33	34	35	36	37
A	S	S	S	V	S	Y	M	H	W	Y	Q
38	39	40	41	42	43	44	45	46	47	48	49
Q	K	P	G	K	A	P	K	R	W	I	Y
50	51	52	53	54	55	56	57	58	59	60	61
D	T	S	K	L	A	S	G	V	P	S	R
62	63	64	65	66	67	68	69	70	71	72	73
F	S	G	S	G	S	G	T	D	Y	T	F
74	75	76	77	78	79	80	81	82	83	84	85
T	I	S	S	L	Q	P	E	D	I	A	T
86	87	88	89	90	91	92	93	94	95	96	97
Y	Y	C	Q	Q	W	S	S	N	P	P	T
98	99	100	101	102	103	104	105	106	107		
F	G	Q	G	T	K	L	Q	I	K		

Length of Sequence : 106 amino acids

Human Framework : 1REI (Bence Jones protein), Human kappa chain group I Plus changes from table 1A and backmutations under BMLr in table 2A

Complementarity Determining Regions (rectangles) : **CDRL1**: L24-34 (10) **CDRL2**: L50-56 (7) **CDRL3**: L89-97 (9)

(CDR definitions and numbering scheme are according to: Kabat *et al.*, 1991)

FIG. 4B FINAL SEQUENCE (INCORPORATING BACKMUTATIONS) FOR HUMANISED ANTIBODY BLC595 V_H (BMHq)

1	2	3	4	5	6	7	8	9	10	11	12
E	V	Q	L	V	E	S	G	G	G	V	V
13	14	15	16	17	18	19	20	21	22	23	24
Q	P	G	G	S	L	R	L	S	C	A	A
25	26	27	28	29	30	31	32	33	34	35	36
S	G	F	T	F	S	**S**	**Y**	**G**	**M**	**S**	W
37	38	39	40	41	42	43	44	45	46	47	48
V	R	Q	A	P	D	K	G	L	E	L	V
49	50	51	52	52A	53	54	55	56	57	58	59
A	**T**	**I**	**N**	**S**	**N**	**G**	**G**	**S**	**T**	**Y**	**Y**
60	61	62	63	64	65	66	67	68	69	70	71
P	**D**	**S**	**V**	**K**	**G**	R	F	T	I	S	R
72	73	74	75	76	77	78	79	80	81	82	82A
D	N	S	K	N	T	L	Y	L	Q	M	N
82B	82C	83	84	85	86	87	88	89	90	91	92
S	L	R	T	E	D	T	A	V	Y	Y	C
93	94	95	96	97	98	99	100	100A	100B	100C	101
A	R	**D**	**R**	**D**	**G**	**Y**	**D**	**E**	**G**	**F**	**D**
102	103	104	105	106	107	108	109	110	111	112	113
Y	W	G	Q	G	T	L	V	T	V	S	S

Length of Sequence : 120 amino acids

Human Framework : 8FAB (Myeloma protein HIL), Closest to human heavy chain group III
Plus changes from table 1B and backmutations under BMHq in table 2B

Complementarity Determining Regions (rectangles) : **CDRH1**: H31-35 (5)
CDRH2: H50-65 (17)
CDRH3: H95-102(11)

(CDR definitions and numbering scheme are according to: Kabat *et al.*, 1991)